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Photophysical characterization of triazole-substituted coumarin fluorophores

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ABSTRACT

The photophysical properties of fluorochromes are directly influenced by their chemical structure. There is increasing interest in chemical strategies that provide controlled changes to the emission properties of biologically compatible fluorophores. One strategy employed is the conversion of a fluorophore-attached alkyne to a triazole through a copper-catalyzed Sharpless-Meldal reaction. In this study, we have examined a series of structurally related coumarin fluorophores and evaluated changes in their photophysical properties upon conversion from alkyne to triazole forms. Ethynyl-coumarin structures showed increases in quantum yield (ca. 1.2- to- 9 fold) and bathochromic shifts (up to 23 nm) after triazole formation. To extend these results, we tested the ability of time-dependent density functional theory (TD DFT) to predict the observed changes in fluorophore absorption properties. We found excellent correlation between the predicted absorption values and experiment, providing a useful tool in the design of new fluorogenic probes.

1. Introduction

The characterization of biomolecular systems has been revolutionized by concurrent developments in fluorescence spectroscopy and biomolecular labeling strategies. The advent of sensitive fluorescence detectors has enabled advances in biological imaging and the emergence of the field of single molecule spectroscopy. Bioconjugate strategies for the ligation of fluorescent labels to biomolecules have become important for biochemical characterization, as they allow detection of these species in complex mixtures [1], and their diversity provides access to previously intractable systems. The specificity of any labeling strategy is critical to success in biological assays, whether used for research, commercial, or therapeutic purposes. Bioconjugate methods have been applied to proteins, nucleic acids, carbohydrates, lipids and cells [2–7]. However, there remains a need for bioconjugate strategies with improved sensitivity and selectivity.

Chemical reactions that are specific for a single functional group in the presence of other biomolecules are termed *bioorthogonal reactions*. Bioorthogonal reactions must be tolerant of aqueous solvent conditions and the presence of functional groups such as amines, alcohols, and carboxylic acids. Successful examples provide non-perturbing chemical handles for the modification of biomolecules in vitro or in vivo with exogenous probes [8]. A currently popular example of this class of reactions is the Sharpless–Meldal reaction, which couples azides and alkynes through a Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition [8–12]. The high selectivity and reactivity of the azide and alkyne groups, mild aqueous reaction conditions, and small size of the functional groups make this reaction ideal for many biological applications [11,13].

Traditionally, the Huisgen 1,3-dipolar cycloaddition between azides and alkynes is performed at high temperatures and long durations [14]. Generally, electron deficient alkynes exhibit good regioselectivity, while other alkynes give mixtures of 1,4- and 1,5-triazole regioisomers [11,14]. The Cu(I)-catalyzed form of the reaction uses much milder conditions and is known as the Sharpless–Meldal reaction [9,10]. The reaction has been employed in numerous biochemical studies to detect binding partners, enzymes and substrates, or engineered proteins [15–18].

The product of the Sharpless–Meldal reaction is a triazole moiety which has been proposed to provide a means to alter the spectral properties of the initial azide or alkyne. Known examples include attachment of the azide or the alkyne to the fluorophore core [19–23]. Subsequent formation of the triazole leads to varying increases in emission intensity and changes in emission wavelength, properties desirable in fluorogenic probes. Combinatorial approaches to identify new fluorophores have been employed to

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identify suitable substrates [19]. It is notable that even small changes to these structures can have a significant impact on the resulting emission properties. For example, switching the attachment point on the fluorochrome from the N1 to the C4 position of the triazole can result in significant differences in emission properties [21].

Substitution of coumarin fluorophores with azide or alkyne groups is known to induce changes in fluorescence properties. The 3- and 7-positions of the coumarin backbone have been shown to strongly modulate fluorescence by affecting the energy of the molecule's two lowest excited states [19,20]. This property has been used extensively for detection of enzymatic activity by substitution at the 7-position, exploiting the increase in emission upon unmasking of a hydroxycoumarin [24–27]. Conjugation of an electron-donating triazole ring to the coumarin backbone at one of these positions causes an increase in quantum yield and a bathochromic shift in emission relative to the starting alkyne [19–22]. This observation was first reported by Sivakumar and colleagues with a series of eight 3-azido substituted coumarins [19]. Zhou and Fahrni examined the influence of triazole formation on a single 7-ethynyl substituted succinic acid coumarin ester [20]. Applications of triazole structures to fluorogenic probe strategies have capitalized on an increase in fluorescence emission to enhance signal to noise in labeling and imaging experiments. Recent examples have favored the use of 3-azido-7-hydroxy coumarin in the labeling of proteins, DNA, and glycoconjugates [19,20,22,28–30]. Although this fluorophore shows a large increase in quantum yield (QY, Φ) upon triazole formation, hydroxycoumarins are environmentally sensitive due to the acidic hydroxyl group [24,31,32]. Therefore, quantitative use of these dyes can be complicated by pH sensitivity to the biological microenvironment [7,24,31]. Ethynyl substitutions of these structures can, therefore, provide an alternative strategy that avoids pH sensitivity [20].

In this study, we undertook a systematic examination of changes to fluorescent properties among a group of related fluorophore backbones. We chose to examine coumarin fluorophores since similar structures have been used successfully in fluorogenic strategies. These derivatives are synthetically accessible by established precedents for introduction of alkyne or azide groups onto the coumarin structure at the 3- [19,29,30], 4-[33] and 7-positions [20]. We sought to examine the modulating effects of conjugated triazole formation on this series of ethynyl-coumarin and benzocoumarin structures for changes in UV–vis absorbance, quantum yield, molar absorption coefficient, and fluorescence emission. Comparison of the experimental data with theoretical calculations of the absorbance properties of the fluorophores allowed us to test the accuracy of electronic models.

2. Results and discussion

2.1. Fluorophore synthesis

Synthesis of the pre- and post-click compounds used in this study was performed using established methods, as depicted in Fig. 1. Coumarin fluorophores were generated by Pechman and Knoevenagel condensations of the corresponding diols, generating four different hydroxycoumarins. To generate the 7-hydroxy-4-methyl coumarin backbone (**1a**, 4-methylumbelliferone), resorcinol (**5**) was reacted with ethyl acetoacetate in a $TiCl_4$-catalyzed Pechmann condensation [34]. Two isomers of benzocoumarin were generated from 2,7-dihydroxynapthalene (**6**) in a sulfuric acid-catalyzed Pechmann condensation with ethyl acetoacetate, providing the angular- (**2a**) and linear-benzocoumarins (**3a**) after purification [35–37]. A second umbelliferone derivative was generated by a piperidine catalyzed Knoevenagel condensation of diethyl glutaconate with 2,4-dihydroxybenzaldehyde to form compound **4a** [38]. The hydroxyl derivatives (**1a**–**4a**) were then converted to the corresponding triflic ethers. The intermediate triflate was used without purification to generate trimethylsilyl-protected alkyne intermediates via Pd(0)-catalyzed Sonogashira coupling with trimethylsilyl acetylene [20,39]. Deprotection with tetrabutylammonium fluoride (TBAF) resulted in the ethynyl compounds (**1b**–**4b**) [20].

With the conjugated alkyne derivatives in hand, we proceeded to generate the click products to characterize their properties. Although an azide coupling partner that is itself conjugated to an aromatic moiety could enhance the fluorogenic properties of the products [19], we chose to use benzylazide to more closely mimic a triazole product that might be formed in a bioconjugate addition. A variety of conditions have been used for similar reactions, with variables including the choice of base/ligand, solvent, reducing agent, and source of copper [14]. The triazolyl-click products (**1c**–**4c**) were generated using two sets of conditions, in the first, Cu(I) catalyzed Sharpless–Meldal reaction with benzylazide was performed using CuI in 1:1 methanol/water with triethylamine as base [14]. These reactions proceeded in good to moderate yields (44–87%). However, we observed minor amounts of the 5,5′-bistriazole products under these conditions, as evidenced by the appearance of a new ^{1}H NMR signal between 4 and 5 ppm and the observation of a peak in the mass spectrum for [$2M^+$-2H] [40]. In one case, we were successful in obtaining crystals of the side product suitable for X-ray diffraction [41]. This contaminant was only observed in detectable amounts in the synthesis of **1c** (23% of bistriazole) and **4c** (7% of bistriazole). We obtained exclusive formation of the desired products **1c** and **4c** with good yields (ca. 62–79%) by altering the conditions used for these substrates to 0.2 equiv. $CuSO_4$ and 0.3 equiv. ascorbic acid in 1:1 methanol/water (Fig. 1). In reactions with equimolar $CuSO_4$ and ascorbic acid at pH 4, we observed the formation of a fluorescent contaminant. The contaminant could be generated in the absence of the alkyne derivative and showed fluorescent emission at 450 nm. The triazole compounds were, therefore, purified by flash chromatography before characterization.

2.2. Fluorophore characterization

To quantitate changes in photophysical properties after triazole formation, we characterized the properties of the hydroxyl- (**1a**–**4a**), ethynyl- (**1b**–**4b**), and triazolyl- (**1c**–**4c**) compounds. These compounds were characterized to determine their UV–vis absorbance spectra, fluorescence emission, molar absorption coefficients, and quantum yields (Table 1). Spectral characterization was performed in ethanol at pH 7 using a quinine sulfate standard in 0.5 M sulfuric acid [31,42]. Our results confirmed that triazole formation generally enhances the fluorescence of alkyne derivatives and can induce shifts in emission wavelength (Table 1). In all four alkynes we observed a large decrease in QY relative to the starting hydroxyl derivatives (ca. 20–90%) with **1b** (from 0.33 to 0.02) and **3b** (from 0.35 to 0.01) showing the most significant changes. Three of the alkyne structures (**1b**, **3b**, and **4b**) show a significant increase in extinction coefficient relative to the hydroxyl derivatives. Conversion of the alkyne structures to the corresponding triazole increased the QY of all structures, with **1c** and **3c** showing the largest relative changes. With the exception of compound **2b**, triazole formation caused a slight decrease in the extinction coefficients as compared to the corresponding alkyne. The increased brightness ($\varepsilon \times \Phi$, $cm^{-1}M^{-1}$) of compounds **1c** and **2c** suggests that these may be the most suitable as fluorogenic probes, while compounds **3c** and **4c** show only minor changes by this analysis. Although relatively large bathochromic shifts have been reported for related compounds, we only observed modest